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CONFERENCE ON PROBLEMS OF GENERAL AND CELLULAR PHYSIOLOGY RELATING TO FERTILIZATION. II.[1]

SOME EFFECTS OF SPERM EXTRACTS ON THE EGGS OF MYTILUS

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THIS is a short progress report on some of the work which I have carried out in the last few months. First, I would like to say a few words about Mytilus eggs as they have been used very little for experimental work and probably not many of you are familiar with them. *Mytilus edulis* is the common mussel that occurs abundantly in the San Francisco Bay. The animals are easily collected and if kept cold over night, they will usually spawn the next day.

Mytilus eggs look somewhat like sea urchin eggs. They are about 65 microns in diameter with closely adhering vitelline membranes, one or two microns in thickness. The membrane does not lift off after fertilization, as in the sea urchin, but remains closely adherent to the surface of the egg. The first cleavage is unequal with a large contact area between the two blastomeres, and consequently the blastomeres are not quite spherical due to this large flat contact area.

[1] Sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949. Conference Chairman, Earl T. Engle, Columbia University, New York. Program Secretary, Albert Tyler, California Institute of Technology. Representative of the N.R.C., Deborah C. Leary, New Haven Hospital, New Haven.

My aim was to separate the blastomeres of early cleavage stages for physiological studies. The presence of the membrane makes it impossible to separate them by using a glass needle and although alkaline sea water will dissolve the membrane, it is injurious to the eggs. Recalling Dr. Tyler's discovery of an egg membrane lysin in Megathura sperm extracts, it occurred to me that such a lysin might be present in Mytilus sperm and this could then be used to remove

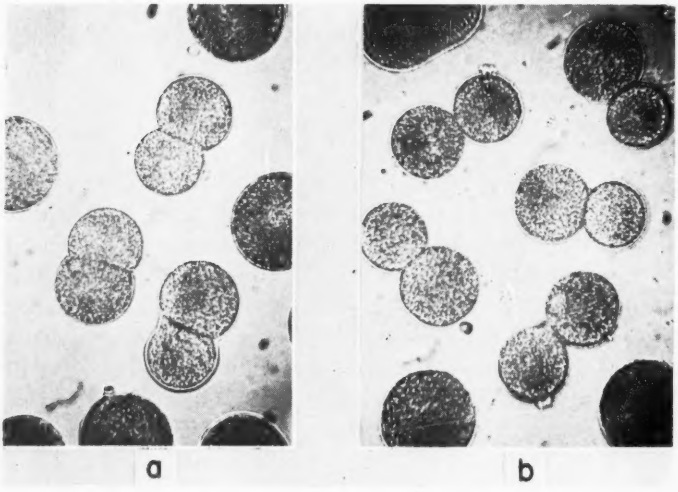

FIG. 1. (a) Normal appearance of blastomeres after first cleavage of Mytilus eggs. (b) Cleavage of Mytilus eggs in sperm extract showing the rounding up of blastomeres and reduction of contact area.

the vitelline membrane. It was discovered that Mytilus sperm also contain a similar enzyme. If the sperm are concentrated, frozen, thawed and then centrifuged, the supernatant contains a substance capable of dissolving the membrane of Mytilus eggs. Ordinarily the membrane is so thin that it is difficult to see whether it has been dissolved; however, if the eggs are first plasmolyzed, the membrane stands out clearly and a sperm extract will cause its dissolution in a few minutes.

Eggs which have had the membranes removed by a short treatment with the sperm extract, however, still cleave in a normal fashion; the blastomeres are in very close contact and it is difficult to separate them. It was discovered that if the cleaving eggs were left in the sperm extract a striking thing happens. *The blastomeres become spherical and many of them become nearly or completely separated.* (See Fig. 1b). The blastomeres of any of the early cleavage stages can be separated by this method apparently without harmful effects. Also the polar lobe may be removed with ease.

In addition to perhaps being of future value as a method for separation of blastomeres, it is also of some interest that the sperm extracts contain a substance which has such an effect on the cleaving egg. The question is whether this "blastomere separating" substance is the same as the sperm lysin. As far as I could determine, the lytic effect of the sperm extract always parallels the blastomere separating effect. Whenever the lytic activity of a particular sperm extract is weak, the blastomere separating effect is also slight. Both effects of the sperm extract are destroyed after heating, although the Mytilus sperm lysin is much more resistant to heat than that of Megathura. There is still a slight activity after 10 minutes at 100° C. Again with heat treatment there seems to be a parallel inactivation of the sperm lysin and the disappearance of the blastomere separating effect. Perhaps the chemical methods used by Dr. Tyler would be helpful in deciding whether one or two substances are responsible for these effects.

It seems most likely that the sperm extract causes a separation of the blastomeres by dissolving the cement between them.

Discussion

Chairman Engle: Thank you, Dr. Berg. Do you know anything about the nature of that membrane?

Dr. Berg: You mean chemical properties? No, I don't, as I haven't carried out any chemical studies on these eggs.

DR. TYLER: Have you tried a hyaluronidase preparation from bull testes or other sources?

DR. BERG: No, I haven't.

DR. TYLER: We have tested it quite extensively on sea urchin eggs without finding any definite effect.

DR. SPIKES: Can you get the same effect by putting eggs in calcium-free sea water?

DR. BERG: Just recently I found if one takes off the membrane, then with the proper treatment with calcium-free sea water they will separate, which suggests again it is the matter of dissolving the cement between the cells.

DR. SPIKES: That would indicate at least two agents in the sperm extract, one for the membrane and one for the cement.

DR. BERG: That was what I was trying to find out, whether there were really two substances there. The only way to answer that would be to try to carry out chemical studies of the sperm extracts.

DR. A. R. MOORE (University of Portland): I noticed there are two types of membrane, the fertilization membrane and the one which clings closely along the blastomeres. Those can't be the same.

DR. BERG: The photographs don't show the membranes, and I believe what appeared to be a membrane was an artefact. Ordinarily it is very difficult to see the membrane as it is transparent and only one or two microns thick. You might be able to make out the edge.

DR. KRAUSS: Dr. Berg, did you form any concept concerning the possible role of this substance in the fertilization of the egg?

DR. BERG: No, I haven't. Of course, as Dr. Tyler has mentioned, the lysin may play a role in allowing the sperm to penetrate through the membrane.

DR. TYLER: For that purpose it would not be necessary to dissolve the membrane completely. In the mammalian egg it has been shown recently that fertilization can be accomplished without any marked dispersal of the cumulus cells or dissolution of the cementing substance that holds

them around the egg. This has inclined some investigators to the view that hyaluronidase is of no particular significance in penetration. I think it is clear, however, that a single spermatozoon can carry enough of the lytic agent to dissolve a path for itself, which may subsequently close over. There is no reason for assuming, at present, any joint action by a large number of sperm.

DR. CHAMBERS: This summer William White studied the action of purified beef testis hyaluronidase supplied by Dr. Duran-Reynals and sea urchin sperm extract prepared according to the method of A. Claude for the isolation of hyaluronidase. He found that the beef testis hyaluronidase had no effect on the extraneous coats. However, the sea urchin sperm extracts used in a dilution of 1 to 1,000 caused a swelling and dissolution of the jelly, but had no effect on the fertilization membrane and hyalin plasma layer.

DR. TYLER: Mr. Krauss and I have examined quite extensively the action of beef hyaluronidase on sea urchin eggs and our results agree with what you report were obtained by Dr. White. On the other hand, the dissolution of the gelatinous coat by a sperm extract, which was also reported last year by Monroy and Ruffe, we find to be interpretable on the basis of contraction of the precipitation membrane to the surface of the egg proper, as is illustrated in Fig. 5 of my paper.

DR. WILLIS PEQUEGNAT: I am interested in the effect on the blastomeres because the inhibitor I mentioned a while ago caused a similar effect in sea urchin eggs. Arbacia eggs were exposed to this substance for a considerable time and then fertilized. They were completely rounded up and sometimes there was complete separation. Also in relation to the corona effect you mentioned, essentially the same thing appeared in these preparations.

DR. BERG: There are other things, such as alkaline sea water, which will also cause these radiations.

DR. GIESE: Did you try the extracts of sperm in other forms?

DR. BERG: The only form I tried besides Mytilus was

Urechis and the results were completely negative. Also Mytilus sperm extract had no effect on Urechis eggs and *vice versa.*

DR. SCHECHTMAN: In Mytilus or other organisms that have been studied for the lytic effect, does the treatment of the egg with the lytic preparation result in a definite removal of the lytic principle? In other words, would eggs subsequently put into the solution be markedly protected?

DR. BERG: I haven't tried it. Perhaps Dr. Tyler has.

DR. TYLER: We have absorption experiments that show lowering of activity of the lytic agent. They are not good enough quantitatively to answer the question of whether or not the eggs can absorb it completely.

DR. SCHECHTMAN: I don't recall any one having found this. Perhaps you have worked on it, but I don't recall. Has any one found that the injection of sperm produces an anti-lytic substance which can remove all the activity?

DR. TYLER: We have made anti-sera against sperm extract containing the lytic agent of Megathura. In fact, anti-sera against inactivated (by heat) extract will inactivate the lytic agent.

CHAIRMAN ENGLE: In place of Dr. Gilbert Smith, who was unable to attend this conference, Dr. Markert, of California Institute of Technology, has agreed to report briefly on a diffusible sex-stimulating substance in a fungus. We shall now hear from Dr. Markert.

SEXUALITY IN THE FUNGUS, GLOMERELLA[1]

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DR. SMITH was scheduled to speak this morning on his studies of the sexual substances of the unicellular alga, *Chlamydomonas,* but since he wasn't able to be here, I should like to say a few words about sexual substances in another plant—the fungus, *Glomerella.* This fungus is a common plant pathogen which produces one of the diseases known as anthracnose. The phenomenon of sexuality in *Glomerella* has been studied by Werner Hüttig (1935) in Germany and by several investigators at Louisiana State University, notably Lucas *et al.* (1944), Edgerton *et al.* (1945), Chilton *et al.* (1945), Lucas (1946), Wheeler *et al.* (1948).

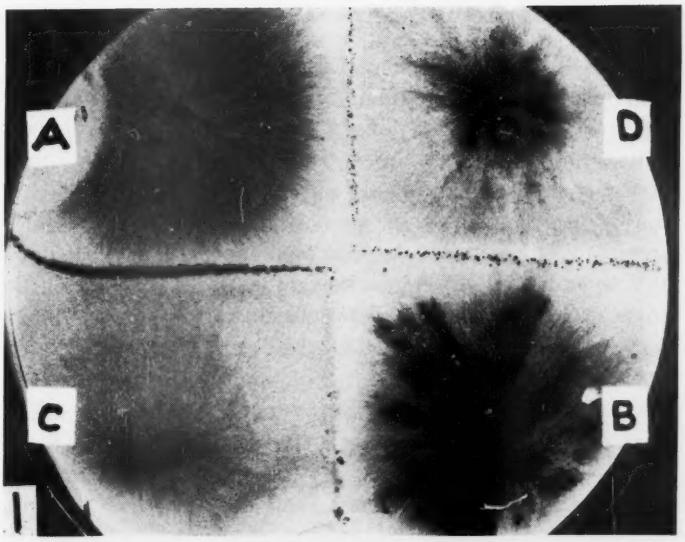
Glomerella exists in a number of different strains which can be distinguished by their different degrees of fertility in sexual crossing. The usual method of demonstrating the mating reaction in *Glomerella* involves the inoculation of a petri dish containing 2 per cent. cornmeal agar (Difco) with the various mating types. When the strains, which are placed at opposite sides of the petri dish, grow together in the center, sexual crossing occurs (see Figs. 1 and 2). This crossing reaction results in the formation of a row of black perithecia—which are spherical reproductive structures containing the ascospores. The significant fact, from the viewpoint of sexuality, is that different strains react with one another to different degrees—that is, they exhibit different

[1] Presented before the Conference on Problems of General and Cellular Physiology Relating to Fertilization, sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949.

degrees of fertility. Environmental factors such as temperature, moisture, nutrition, etc., also affect the number of perithecia which are formed at the line of juncture between two mating types. Nevertheless, when environmental conditions are controlled, the number of perithecia formed by any two strains is constant enough to be a reasonably good measure of their mutual fertility.

The strains of *Glomerella* may be divided into two basic groups: (1) those that produce perithecia homothallically, and (2) those that do not. In general, almost any two strains of *Glomerella* will cross with one another to some extent, provided that at least one of the strains carries the factor for perithecia formation. This brings me to the first general principle that I should like to mention—namely, the fertility of any cross depends upon complementary genetic factors in each strain. Thus, no one strain is more fertile than all the others in all possible crosses. For example, in comparing the fertility of strains *C* and *D*, we find that *C* is the more fertile in crosses with strain *A*, but that *D* is the more fertile in crosses with another strain *B* (see Fig. 1).

The second general principle which I want to bring out is that apparently the fertility of the cross between two strains is dependent upon some diffusible chemical substance. This principle was demonstrated in the following way: A petri dish was prepared with two layers of cornmeal agar separated by a cellophane membrane. Inoculated on the top layer of agar were strains *A* and *D* which normally cross weakly with one another. Underneath the cellophane membrane was inoculated strain *C*, which normally crosses very strongly with strain *A*. Strain *C* grew throughout the agar beneath the cellophane membrane but did not penetrate the membrane. When strains *A* and *D* finally met in the middle of the petri dish they crossed very strongly as shown by the formation of a dense row of perithecia. In fact, the cross between *A* and *D* seemed stronger under these circumstances than the normal cross between *A* and *C*, which is a very strong cross, indeed. It is apparent, there-



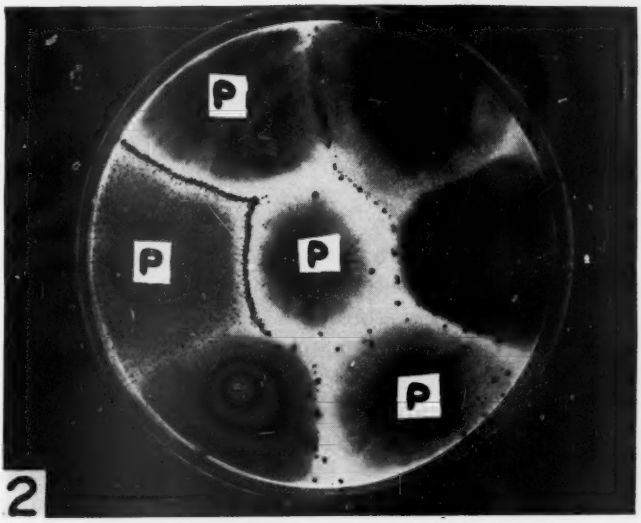


FIG. 1. The magnitude of the crossing reaction between strains A + C, B + D, A + D, and B + C is dependent upon the possession of complementary genetic constitutions by the crossing strains. Consequently, no single strain can be described as more fertile than all other strains.

FIG. 2. Examples of the crossing reaction between various strains of *Glomerella*. The strains labeled *P* (perithecial) form perithecia homothallically and also by sexual crossing with adjacent strains.

fore, that some substance produced by strain *C* diffuses through the cellophane membrane and stimulates the crossing reaction between strains *A* and *D*.

In summary, then, the two important principles illustrated by these experiments on *Glomerella* are, first, that the degree of fertility between the various strains of this organism is controlled by complementary genetic factors, and second, that these genetic factors exert their effects in part, at least, by means of diffusible substances. These same principles may, of course, apply also to higher organisms.

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DISCUSSION

CHAIRMAN ENGLE: That is a very interesting point. You are continuing with these experiments, I take it.

DR. MARKERT: Yes, these are only preliminary experiments. Further experimentation may of course modify these conclusions somewhat.

DR. TYLER: You avoided using the term "relative sexuality."

DR. MARKERT: I do not believe the concept of relative sexuality is applicable to *Glomerella* because no single strain is uniformly more fertile than all the others in all crossing combinations. Furthermore, the strains of *Glomerella* can not really be divided into two sexes, since in general almost all strains will cross with all other strains, including itself, provided that at least one of the strains carries the

factor for perithecia formation. Hartmann's concept of relative sexuality was applied to *Glomerella* by Hüttig (1935). However, Hüttig's use of this concept was probably based upon the study of an insufficient number of strains of *Glomerella*. A few strains can be selected which do fit the concept of relative sexuality, but when all strains are considered some other concept becomes necessary to explain their sexual behavior.

DR. SCHECHTMAN: I wondered if you had tried, let us say, *A* and *C* on top and *D* below. In other words, would there be evidence of inhibiting diffusible substances?

DR. MARKERT: I tried the particular combination you mentioned. There was no inhibition. However, I haven't tried all possible combinations, and some strains may yet be found which do exert an inhibiting effect upon the crossing reaction between other strains. Whether there are many strains which can promote the crossing reaction between other strains, I do not know.

THE RELATION OF IONS TO THE APPEARANCE AND PERSISTENCE OF THE FERTILIZATION AND HYALINE MEMBRANES IN THE EGGS OF THE SEA URCHIN[1]

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and
Hopkins Marine Station, Pacific Grove, California

The elevation of the fertilization membrane is the first clear evidence of fertilization in the echinoderm egg. As soon as this membrane has reached its full extension, the hyaline layer appears on the surface of the egg and continues thickening, reaching its full form, 2.4 micra thick, in 15 to 20 minutes at 20° C. These two structures surround the dividing egg, the fertilization membrane serving as a cushion for the early embryo, providing a milieu of comparative constancy, while the hyaline layer holds the blastomeres together so that, as a result of their divisions, they form a hollow sphere.

The following discussion concerns the questions of the origin and nature of the fertilization membrane and of the hyaline layer. In order to throw light on these matters it may be helpful to consider an experiment published some years ago which showed that if the unfertilized eggs are first put into an isosmotic solution of urea or other non-electrolyte and, after a minute or longer, are removed to sea water, they can be fertilized, but no membranes form (Moore, 1930). Segmentation occurs in normal fashion, the cell-divisions taking place after the same time intervals as in

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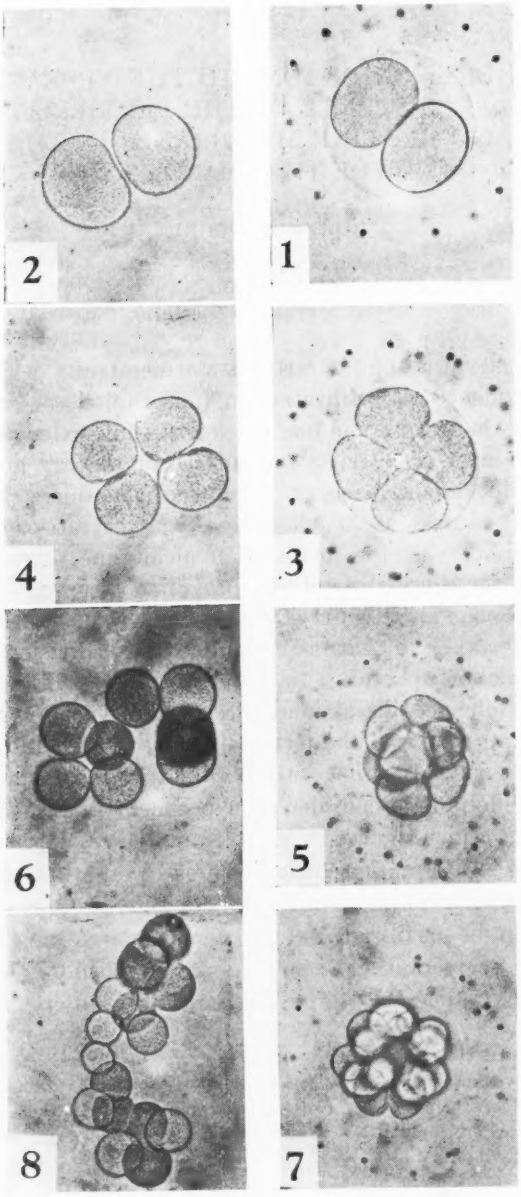
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the untreated controls (Figs. 1–8). This is sufficient to show that criticism of the experiment to the effect that cleavage is abnormal in these membrane-free eggs (Moser, 1940) is unfounded in fact. The only abnormality the embryos exhibit is the direct consequence of the lack of membranes, as a result of which the blastomeres form plates or cups instead of closed blastulae.

As to why eggs which have been immersed in a solution of non-electrolyte fail to form membranes after having been returned to sea water and fertilized, I have suggested that the pre-membrane material is dissolved out by any solution which lacks cations in a certain concentration and of which the pH is greater than 5. The facts which support this view have been made clear in a series of experiments with the eggs of a number of species of sea urchins (Moore, 1935a) in which it has been shown that the preservation of the membrane-forming function of the egg depends upon the pH and the concentration of the metal ions in the solution surrounding the eggs. For example, in a solution of glycerol it was found that the effectiveness of the solution in destroying the pre-membrane system fell off only slightly as the pH varied from 9 to 5, but that between 5 and 4 it fell to zero (Moore, 1932a) (Fig. 8a). Thus the membrane-forming capacity of the egg is not destroyed in the absence of metal ions if the pH is 4 or lower. It must therefore be concluded that the active agent which prevents membrane formation, *i.e.*, destroys the pre-membrane stuff, is the hydroxyl ion.

It can be shown that the cations of inorganic salts antagonize this destructive action of the hydroxyl ion, for if a given quantity of inorganic salt be added to the solution of non-electrolyte, it protects the membrane-forming capacity of the eggs even in alkaline solutions. The series (Table 1)

FIGS. 1, 3, 5, 7 show normal cleavage in the egg of *Dendraster excentricus*. The first four divisions. FIGS. 2, 4, 6, 8 show cleavage of a membrane-free egg from the same female. The times of division are exactly the same as in the normal. Note that, except for the membranes, the blastomeres are entirely normal in appearance.

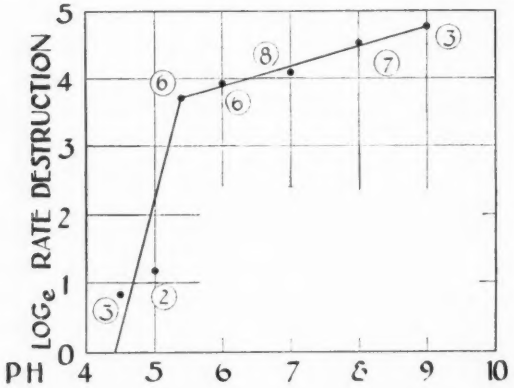


FIG. 8a. Showing the effect of pH on the rate at which the membrane-forming function of sea-urchin eggs is destroyed in an isosmotic solution of glycerol. Rate $= \frac{1000}{\text{time in seconds}}$. The numerals in small circles denote the number of observations from the average of which is derived the adjacent point on the graph.

shows that while a given minimum concentration of the salt varies among the species, it is constant for a given species. Thus, in the eggs of Temnopleurus the concentration for the chlorides of the lithium series is approximately 0.235 M,

TABLE 1

EACH NUMBER DENOTES THE MOLECULAR CONCENTRATION OF THE CORRESPONDING SALT, GIVEN IN THE LEFT-HAND COLUMN, WHICH IS JUST SUFFICIENT IN A SOLUTION OF UREA M/1 AT PH 8, TO PROTECT THE MEMBRANE-FORMING CAPACITY OF 50 PER CENT. OF THE EGGS. CO DENOTES COBALTAMINE CHLORIDE AND THE NUMBER AFTER IT THE VALENCE. THE MONOVALENT COBALTAMINE CHLORIDE, SINCE IT WAS WITHOUT EFFECT, IS OMITTED FROM THE TABLE

	Clypeaster japonicus	Temnopleurus hardwickii	Pseudocentrotus depressus	Strongylocentrotus pulcherrimus	S. nudus	Salt avg.	Average effectiveness numbers
LiCl	.....	0.21	0.10	0.17	0.24	0.18	5.5
NaCl	0.25	0.21	0.10	0.20	0.22	0.19	5.3
KCl	.....	0.28	0.10	0.13	0.25	0.19	5.3
RbCl	.....	0.22	0.12	0.17	0.25	0.19	5.3
CsCl	.....	0.25	0.12	0.17	0.25	0.20	5.0
Species average	0.25	0.235	0.11	0.17	0.24	.....	.....
$MgCl_2$	0.0074	0.0068	0.0038	0.0045	0.0069	0.0059	169.5
$CaCl_2$	0.0060	0.0042	0.0023	0.0037	0.0028	0.0038	263.1
$SrCl_2$	0.0064	0.0068	0.0038	0.0030	0.0046	0.0049	204.0
$BaCl_2$	0.0042	0.0046	0.0023	0.0020	0.0036	0.0034	293.8
Co_2	0.0025	0.0020	0.0015	0.0020	0.0020	0.0020	500.0
Co 3	0.00025	0.00004	0.0002	0.0001	.....	.....	.....
Co 4	0.00020	.....	.....	0.0001	.....	.....	.....
Co 6	0.00005	.....	.....	0.00002	.....	.....	.....

but in Pseudocentrotus it is 0.11 M and in *Strongylocentrotus nudus* it is 0.24 M and very nearly the same for *S. purpuratus*. From these facts it must be concluded that the protective effect of the monovalent series is a function of the valence of the cation, and that there is no specific effect of a particular cation. But with the divalent series of metals another factor appears in that there are differences between specific cations. Thus while Ca is 50 to 100 times as potent in its action as Na, the ion Mg is always much less effective than Ca; while Sr and Ba approximate Ca in their effectiveness. It is clear that something other than valence plays a part, some specific property of the divalent ion. It should also be noted that unlike many other physiological reactions there is no evidence of antagonism between mono- and di-valent cations; here their action is synergic (Moore, 1932b). In this there is a striking parallelism between the effects described for sea urchin eggs and those discovered by Gray (1931) for the solubility of the intercellular matrix of the ciliated epithelium of the gills of Mytilus. Gray sums up the matter thus: "The real dispersive agent is the hydroxyl ion since this ionizes the matrix. All salts inhibit the process, divalent ions being much more powerful than the monovalent cations."

However, the effect of increasing the valence is so striking in the case of the cations, that it seemed important to determine whether the valence of the anion also might possibly be a factor. During the past season a series of experiments has been carried out by R. S. Dean and myself for the purpose of testing the matter (Table 2). The question received a clear answer, which is indicated by the two salts of Mg,

TABLE 2

THE CONCENTRATIONS OF SALTS EFFECTIVE IN PROTECTING THE MEMBRANE-FORMING CAPACITY OF THE EGGS OF *Strongylocentrotus purpuratus*

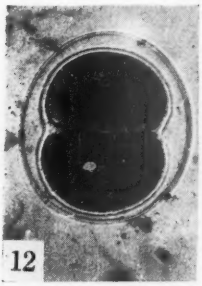
Li Cl	0.2 M	Na_2 oxalate	0.12 M
Na Cl	0.2 M	Na_2HPO_4	0.11 M
KCl	0.2 M	Na_2SO_4	0.10 M
		Na_2 tartrate	0.10 M
$MgCl_2$	0.017 M	Na_3 citrate	0.07 M
$CaCl_2$	0.004 M	$K_4Fe(CN)_6$	0.10 M
$SrCl_2$	0.007 M		
$MgSO_4$	0.017 M		

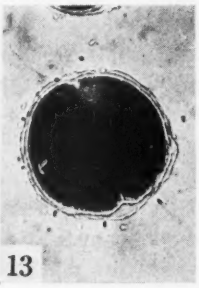
the chloride and the sulfate, in that their effective concentrations are identical. The difference in the valence of the anions has no effect on the minimum concentrations. Na_2SO_4 is twice as effective as the chloride of the same molecular concentration, and the Na_3 citrate is three times as effective, results to be expected if the dissociation of the salts is complete, indicating that the effect depends on the normality of the solution. $K_4Fe(CN)_6$ is, however, only twice as effective as the chloride of the same molecular concentration. These results demonstrate that multiple valences of the anions are without effect on the reaction; only in the case of the cations is valence important. When it is remembered that the tetravalent cobaltamine chloride is 1000 times as effective as the monovalent cation, the failure of the 4-valent anion $Fe(CN)_6$ to affect the reaction is evidence as clear-cut as it could well be that the valence of the anion is of no moment in this reaction.

In considering these quantitative aspects of the protective action of the cation we left aside for the moment the question of how it is that in so brief a time as 30 seconds a solution of urea, for example, can render the egg incapable of forming a membrane. That the initial action of the urea solution is extremely rapid is shown by Motomura's (1934) experiment in which the eggs are exposed to a solution of non-electrolyte for 6 or 7 seconds, then returned to sea water in which the normal fertilization membranes soon appear, an instance of artificial parthenogenesis (Fig. 9). As in some other cases of artificial parthenogenesis, the membrane appears, not in the activating solution, but after the eggs are returned to sea water. In recent experiments with the eggs of *S. purpuratus* I have found that the action of the urea solution is extremely rapid, less than 2 seconds' immersion is necessary at 2° and 20° C. to activate the egg. This indicates a temperature coefficient near unity and suggests that a physical process, such as solubility, constitutes a sort of trigger action for initiating the formation of the membrane. The other possibility is that the time required for the reaction is a fraction of a second. This would neces-

sitate the observation of a temperature-differential of rate in less than the shortest time necessary for an observation, and this difficulty has so far not been surmounted.

The role of the ions in forming the fertilization membrane and maintaining its integrity is made clear by an





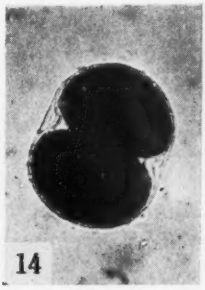


FIG. 9. Normal fertilization membrane and hyaline layer formed by Motomura's method in *Strongylocentrotus purpuratus*. FIG. 10. Same but with fertilization membrane crumpled by the addition of 10 drops of 1 per cent. gelatine to the sea water in which the egg lies. FIG. 11. Promembrane called forth in a solution of urea containing gelatine. This structure, unlike the fertilization membrane, is not crumpled by protein in the sea water about it. FIG. 12. Egg of *S. purpuratus* which has been exposed to a solution of M urea containing NaCl in 0.25 M concentration, and then fertilized. 2-cell stage, perfectly normal. FIG. 13. Same, but with the urea solution containing 0.12 M NaCl. The fertilization membrane has been formed following fertilization but not elevated. FIG. 14. Same, but with the urea solution containing 0.06 M NaCl. The fertilization membrane is absent, but the hyaline layer is complete.

experiment in which the eggs are transferred to a urea solution at intervals after being fertilized in sea water. If such eggs are put into the urea bath 30 seconds after fertilization, both fertilization and hyaline membranes fail and the eggs proceed to divide into blastomeres which form chains; after 45 seconds, fertilization membranes are present but fragile and much distended, quite abnormal. Even after 3 minutes, when the membrane is fully formed and elevated, immersion in the ion-free solution causes the structure to be weakened and distended. It is only when the membrane is 4 or 5 minutes old that it is able to resist the dissolving effects of a salt-free solution.

On the question of the origin of the fertilization membrane, there are two hypotheses, namely, either the membrane is formed by the interaction of pre-membrane substances in the cortex following the entrance of the sperm, or is already formed on the surface of the unfertilized egg, activation serving only to lift it. That the fertilization membrane is a structure strikingly different from the membrane on the unfertilized egg is shown by the following facts obtained from experiments on the eggs of *S. purpuratus:*

(1) The fertilization membrane at first is fragile and elastic, dissolving in an ion-free solution. In sea water, however, the membrane develops, during the first 4 or 5 minutes, its characteristic toughness and rigidity. One element, therefore, in the maturation of the membrane consists in the salts of sea water. E. N. Harvey has suggested that oxidation may play a part as it does in the hardening of the silk fiber, on exposure to air, after it leaves the body of the worm.

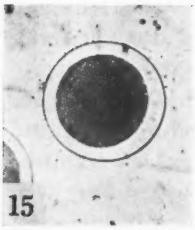
(2) If it is assumed with Runnström that before fertilization the membrane lies on the egg as a curved sheet consisting of folded protein chains, and that the effect of fertilization is an unfolding, and hence enlargement of the structure, we should expect that, when osmotic pressure is applied by means of large protein molecules in the surrounding solution (Dean and Moore, 1947), the resulting shrinking of the membrane would take place in a regular

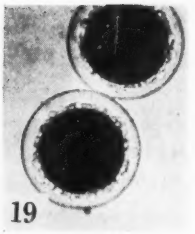
pattern of wrinkling. This does not occur, but instead the crumpling is entirely irregular, giving no indication of a previously existing wrinkle pattern (Fig. 10).

If we take the view rendered probable by these considerations, namely, that the fertilization membrane exists, not as a formed membrane on the unfertilized egg, but as a gen or precursor in the cortex of the egg, then we must suppose that the precursor substance or substances leave the egg during the 60 seconds or less that the egg remains in the solution of non-electrolyte, as described in the foregoing experiments. The failure of the fertilization membrane to appear on eggs treated in this way when they are returned to sea water and fertilized, as stated above, has been ascribed to the action of the hydroxyl ions in dissolving out the premembrane material from the cortex of the egg (Moore, 1935b). In the earlier experiments the loss of substance from the egg escaped visual detection. I have found, however, that if a little protein, gelatin or albumen be added to the salt-free solution, the loss of material from the egg becomes at once apparent. This pre-membrane stuff appears in two stages. The first, which we may call promembrane A, is seen as a clear membrane pushing rapidly out of the egg in the first few seconds after immersion. The second, pro-membrane B, follows in approximately 10 seconds, and appears as a denser, more refractive zone within the first (Fig. 11). The boundary of B never overtakes that of A. Both extend enormously, far beyond the limits of a normal fertilization membrane (Figs. 16, 17). This fact of the extensibility of the pro-membrane emphasizes the remarkable property of the fertilization membrane in its precisely limited extension, when completely formed. The protein nature of A and B is shown by the fact that they quickly disappear in a solution of urea containing trypsin. This establishes a point of identity as to chemical constitution between promembranes and newly formed fertilization membranes which also dissolve in sea water containing trypsin.

A short time after the eggs which have been bathed in a

solution of non-electrolyte are immersed in sea water and the pro-membranes dissolved, a third substance, a clear granular mass exudes from the egg (Fig. 18). Its timing and reactions indicate that this substance may be considered the stuff which, under normal circumstances, makes up





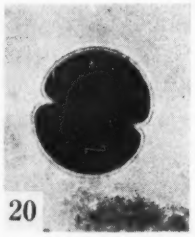


FIG. 15. Normal fertilization membrane in an egg of *S. purpuratus*. FIG. 16. Beginning of promembranes A and B 10 seconds after putting the egg into 10 cc. of solution of urea M/1 + 0.5 cc. 1 per cent. gelatine. FIG. 17. Same 30 minutes later. Note extension and the fact that the two protomembranes do not coalesce. FIG. 18. The egg was returned to sea water after the formation of protomembranes A and B. These quickly dissolve and in a few minutes clear granules come out of the egg, forming a mass which reacts in acid water and in a solution of non-electrolyte, exactly like the hyaline layer. Hence the exudate may provisionally be called prohyaline substance. FIG. 19. Fertilized eggs of *S. purpuratus* put into sea water which has been rendered acid to pH 3.5. Here the hyaline layer loses its structure and forms droplets. This layer behaves as if it were a calcium proteinate. FIG. 20. Egg which has been put into trypsin sea water 2 minutes after fertilization. The newly forming fertilization membrane has been dissolved by the trypsin, but the hyaline layer remains intact in the trypsin sea water solution.

the hyaline layer, for it is soluble in acid sea water (Fig. 19), in M/2 NaCl and in salt-free solutions. It thus acts in all ways like the hyaline layer of the developing egg and may be referred to as the pro-hyaline substance.

We are now in possession of sufficient facts to give a provisional account of the loss of materials by eggs subjected to treatment with a solution of non-electrolyte. In this bath the unfertilized egg excretes the precursor substances necessary for building the fertilization membrane. These substances are the pro-membranes A and B, which, when the eggs are immersed in sea water, quickly dissolve, and the stuff which, in the normal egg, constitutes the hyaline membrane, exudes as an unformed mass. As a consequence, after fertilization, eggs which have been treated in this way develop without supporting membranes. The blastomeres are held together only by cell connectives, and form irregular clusters or plates.

Further information on the nature of pro-membranes A and B may be obtained in another way, namely, by trypsin digestion before fertilization.[2] In order to demonstrate this effect, the unfertilized eggs are put into a solution of trypsin in sea water (100 mg. per cent.) for a certain length of time, and are then transferred to sea water and fertilized. In those which have remained 30 seconds in the trypsin bath before fertilization, normal membranes may develop; on those removed from the bath after 60 seconds, membranes are formed, but not elevated, with the result that the embryos are nearly solid blastulae; after 2 minutes in the trypsin bath no membranes are formed, the blastomeres being held together by the hyaline layer (*cf.* Figs. 12, 13, 14). The experiment shows that the premembrane material in the cortex is dissolved by the trypsin within 2 minutes, and that the normal elevation of the membrane depends upon the presence, between the membrane and the

[2] In a personal communication, September, 1932, Dr. Kunitz told me of experiments with trypsin which he had carried out on eggs of Arbacia. From the results he concluded that "trypsin has an effect on the precursor of the fertilization membrane, but does not affect the membrane once it has (fully) formed."

egg, of protein molecules which are dissolved before those which go to form the membrane. It should be noted that, like the action of ion-deficient solution on the premembrane system, the digestion of the precursors in the egg takes place by steps.

Another method of studying the chemical constitution of the membrane is to submit the fertilized eggs to a trypsin bath after they have been fertilized. If put into this bath 10 seconds after fertilization they subsequently form, in sea water, chains of blastomeres; put into the trypsin bath 2 or 3 minutes after fertilization, the fertilization membrane is dissolved, but the hyaline membrane remains unaffected, holding the blastomeres together to form a blastula. This development may take place even if the eggs remain in the trypsin bath. The fertilization membrane thus readily undergoes the digestion characteristic of proteins, while the digestion of the hyaline layer takes place slowly if at all. Runnström (Runnström, Monné and Broman, 1944) reports that he met with no difficulty in dissolving the hyaline layer with trypsin. This is not unqualifiedly true in my experiments for all concentrations of trypsin. Even in eggs divested of the fertilization membranes, lying in the trypsin bath, the hyaline layer persists (Fig. 20). Thus the newly formed fertilization membrane is more soluble in trypsin than is the hyaline layer. However, if the trypsin solution is used in higher concentration, say 400 mg. per cent., the hyaline layer also is dissolved. It is interesting to compare the results of trypsin digestion in different places with different forms. Kunitz and later Chambers and White (1949) found that in Arbacia the precursor is dissolved by trypsin. This is identical to Runnström's report for two different species on the Swedish coast and to my results with *S. purpuratus*. Therefore, with respect to the trypsin solubility of precursor system, all agree. But in disagreement with Runnström, I have found the fertilization membrane, often up to 5 minutes after fertilization and in one lot of eggs up to 30 minutes, is dissolved by trypsin. The fertilization membrane is therefore protein in its constitution.

That the fertilization membrane differs unmistakably from its precursors is made clear by the following table of properties:

Fertilization membranes	Pro-membranes A and B
1. Early solubility in ion-free solution.	1. Insoluble in ion-free solution.
2. Insoluble in sea water.	2. Soluble in sea water.
3. Rigid when set.	3. Not rigid.
4. Collapsed by small amount of protein.	4. Not collapsed by small amount of protein.
5. Dissolved by trypsin in early stages.	5. Dissolved by trypsin in all stages.

Conclusions

The unfertilized egg contains in its cortex two substances, pro-membranes A and B which, if released simultaneously, react to form the fertilization membrane and to provide for its elevation. This release suggests a trigger reaction, normally set off by the entrance of the sperm, but which may be tripped by any one of the numerous parthenogenetic agents. The reaction can be completed only in the presence of salts, *i.e., cations*. In the case of exposure of the unfertilized egg to non-electrolyte solution, A is followed by B after an interval. As these structures extend, the boundary of B approaches that of A but never overtakes it. At least two factors thus seem to be necessary for the formation of the fertilization membrane:

(1) The very nearly simultaneous release of A and B.

(2) The presence of salts. The facts of Motomura's experiment suggest that salts are essential in forming the membrane.

It is suggested that the origin of A and B may be the granules of the cortex described by Moser (1939). The difference in time of appearance in the case of the two precursors may be referred either to a difference in the rates of solubility or to a spatial difference in the distribution of the substances, *i.e.*, the granules destined to form B may be deeper lying. The result in either case would be temporal and spatial differences in the appearance of A and B which

would mean that there must be at least two types of granules, with possibly a third to account for the pro-hyaline material, and a fourth to provide the large molecules for the elevation of the membrane by osmotic pressure.

The relation of A and B and salts to the formation of the membrane suggests that the mechanism may be analogous to the clotting of blood or milk. In the latter two systems the precursors exist side by side without interaction until a mobilizing factor by a sort of trigger action changes the precursors into reactive forms and the result is a water-insoluble clot. In the egg the fertilization membrane is the final result.

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Discussion

Chairman Engle: Thank you, Dr. Moore. We shan't be able to give the time for discussion that it deserves, and due to that lack of time will the discussants make their comments and questions short.

Dr. Chambers: I was wondering whether in Strongylocentrotus there is a very specific action on the membrane relative to monovalent ions. By putting eggs into the salt

shortly after fertilization the membrane is actually formed. You can't see it, but you can demonstrate it with a needle. As soon as you add strontium it becomes a definite structure. Do you think calcium and strontium could combine with it in a more physical manner than the sodium?

DR. MOORE: That might serve to account for this great difference between calcium and strontium on the one hand and the other salts. I think that your suggestion is a reasonable one.

CHAIRMAN ENGLE: Are there any other comments?

DR. CHAMBERS: I would like to say something about the effect of urea. If we put Lytechinus eggs into urea and then into sea water, the nucleus moves to the center and starts to divide. We tried to see what ions are effective in bringing this about. Sodium was most effective. If we put them into isosmotic solutions of magnesium iodide, this result was not obtained.

DR. MOORE: It is possible your potassium-calcium ratio in the concentrations that are present in sea water could be the cause. Could you reverse the procedure? Could you take them out of the pure salt solution and put them into sea water and fertilize them?

DR. CHAMBERS: I never tried that.

DR. MOORE: It should be pointed out that sodium is the least toxic of the salts of sea water, whereas potassium is very toxic. This fact might account for the effect you describe.

DR. TYLER: Costello has recently described, in eggs of Nereis, an interesting separation of the membrane into two parts. When the eggs are placed in alkaline sodium chloride around pH 10, the membrane swells up, or probably the material under the membrane swells, and then, later the membrane splits into two parts.

DR. MOORE: That is apparently a later separation and a different phenomenon. The separation I have described is most marked at the start, and diminished later.

THE PHOSPHORUS COMPOUNDS OF SEA URCHIN EGGS AND THE UPTAKE OF RADIO-PHOSPHATE UPON FERTILIZATION[1, 2]

DR. ARTHUR H. WHITELEY

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EXTENSIVE work with invertebrate embryos, reviewed by Tyler (1942), and more recently with amphibian larvae by Barth and Jaeger (1947) and Hall and Moog (1948), has clearly shown that many facets of growth and differentiation are closely dependent on energy derived from respiratory metabolism. One of the primary problems of developmental physiology which grows out of these findings is to determine how energy is mobilized from substrates and transferred for use by the specific developmental process. The problem is analogous to that of energy transfer in muscle contraction, and, as in this instance, it is possible that phosphorylation and production of high-energy phosphate will play a central part. In a less direct manner, phosphorus must have an important role in genetic and cytoplasmic control of cellular processes in the embryo, because of its presence in desoxyribonucleic and ribonucleic acids, and recently evidence has been presented that involves certain phosphorylated compounds in control of

[1] The author's contributions described in this article were made in 1946–1947 during the tenureship of a National Research Council Fellowship in Zoology at California Institute of Technology in the laboratories of Dr. Albert Tyler.

[2] Presented before the Conference on Problems of General and Cellular Physiology Relating to Fertilization, sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949.

TABLE 1

PHOSPHORUS COMPOUNDS IN UNFERTILIZED ECHINODERM EGGS

(The data in this table have been recalculated, and in some instances the compounds identified more specifically than by the original investigators)

Phosphorus fraction	*Patiria miniata*	*Dendraster excentricus*	*Paracentrotus lividus*	*Brissopsis*	*Arbacia punctulata*	*Arbacia punctulata*	*Strongylocentrotus purpuratus*	*Lytechinus pictus*
Total P	6.64 mg./gm. dry weight	6.94 mg./gm. dry weight	134.7 mg./gm. N	.....	130 mg./gm. N 3.1 mg./gm. wet weight [4]	960 μg/10^6 eggs [1] 4.13 mg./gm. wet weight [2]	817 μg/10^6 eggs 2.94 mg./gm. wet weight	2.78 mg./gm. wet weight
Acid Insoluble P	48%	50%	53.0%	.....	62.4%	62.2%	69.1%	56.8% [3]
RNA P	16%	32%	30.5%	.....	4.6%	22.2%	38.0%	24.7%
DNA P				.....	1.1%	0.9%	1.7%	0.5%
Phosphoprotein P	trace	12%		.....	19.1%	2.6%	2.4%	1.3%
Phospholipid P	32%	6%	22.5%	.....	37.6%	36.5%	27.0%	20.5%
Acid Soluble P	52%	47%	47.0%	.....	37.6%	37.8%	30.3%	38.2%
Inorganic P	20%	29%	28.7%	53% of ASP.	11.2%	.....	7.4%	15.1%
ATP + ADP P	13%	14%	11.4%	31% " "	21.3%	.....	8.2%	13.8%
Propanediol phosphate P	19%	4%	7.6%	16% " "	.....	.....	12.0%	7.3%
Unidentified P	....	....	.....	.....	5.1%	.....	2.7%	2.0%
Investigator	Needham and Needham (1930)		Zielinski (1939)	Lindberg (1943)	Crane (1947)	Schmidt *et al.* (1948)	Whiteley	Whiteley

[1] Schmidt *et al.* reported 96×10^{-3} μg/egg, but it is believed this should have read 96×10^{-5} μg/egg.

[2] Calculated from the per-egg basis using conversion data from Harvey (1932).

[3] 9.8 per cent. lost in fractionating acid insoluble components.

[4] Crane reports "0.31 milligrams per cent of wet weight."

morphogenesis in the echinoderms. It is the intent of this paper to review the compounds of phosphorus that have been identified in echinoderm eggs from the point of view of these three major uses.

Since the first relatively complete study of the phosphorylated compounds of echinoderm eggs by Needham and Needham (1930), analyses of various species have been published by Zielinski (1939), Lindberg (1943), Crane (1947), and Schmidt, Hecht and Thannhauser (1948). The data are gathered together in Table 1, along with figures obtained for the unfertilized eggs of *Strongylocentrotus purpuratus* and *Lytechinus pictus* by the author. There is relatively good agreement in the kinds and quantities of substances present despite differences in technique and species of sea urchin. Further refinement, and possible identification of additional compounds, will probably depend on development of more specific and accurate methods, such as the enzymatic methods of Kalckar (1947) for the phosphorylated adenosine compounds.

It is interesting that the greatest bulk of phosphorus is contained in ribonucleoprotein and phospholipids, while, in most instances, comparatively little phosphoprotein exists. The echinoderm egg is quite different, in these respects, from amphibian and chicken eggs which contain comparatively little ribonucleoprotein and a large amount of phosphoprotein (Needham, 1942). To date, only three acid-soluble phosphates have been isolated from the eggs of sea urchins: inorganic orthophosphate, propanediol phosphate, and adenosine triphosphate. In addition, three coenzymes have been measured manometrically in the unfertilized eggs of *Arbacia punctulata*. Diphosphothiamine is reported present to the extent of 2.3 micrograms per gram wet weight, or 10.0 micrograms per gram dry weight by Krahl, Jandorf and Clowes (1942), a figure confirmed by Goldinger and Barron (1946) who have found 12.9 micrograms per gram dry weight. Diphosphopyridine nucleotide is present to the

extent of 250–500 micrograms per gram wet weight in the same eggs, approximately 25–40 per cent. in the dihydroform (Jandorf and Krahl, 1942), and Krahl, Keltch and Clowes (1940) find an average concentration of flavin dinucleotide of 9.8 micrograms per gram wet weight. The sum of the phosphorus in these compounds would, to a first approximation, amount to about 2 per cent. of the total egg phosphorus, and could account for the bulk of the unidentified acid soluble phosphorus of *S. purpuratus* and *L. pictus* eggs (Table 1).

It is noteworthy that neither phosphoarginine nor phosphocreatine have been found in mature eggs, though both are present in adult tissues and at least phosphoarginine has been identified in the ovaries of *Brissopsis* (Lindberg, 1943).

Nucleic Acids

The large content of ribonucleic acid in sea urchin eggs is a feature they have in common with a number of invertebrate eggs. Brachet has proposed that this acid serves as a sort of reservoir of material to be converted into desoxynucleic acid as development proceeds and the number of nuclei increases. This view is thoroughly reviewed in his recent book (1945). It has been challenged by Schmidt, Hecht and Thannhauser (1948) on the basis of their finding that ribonucleic acid content remains constant through pluteus formation in *A. punctulata* while desoxynucleic acid increases markedly. Recently Villee *et al.* (1948) have shown that the radiophosphate incorporated into the acid insoluble fraction in *Arbacia* embryos is found in large part in desoxynucleic acid, with very little appearing in the ribonucleic acid. Moreover, malononitrile and other inhibitors depress ribonucleic acid synthesis in these eggs more than desoxynucleic acid synthesis. All these observations remove the force of Brachet's suggestion and lead one to look for a function for this large amount of ribonucleic acid elsewhere than as a precursor of desoxynucleic acid. It is not

unreasonable to suppose that this function is concerned with differentiation of new proteins in the embryo, in view of the arguments brought forth by Brachet (1945) and by Caspersson (1947) implicating ribonucleic acids in protein synthesis.

Phosphoprotein and phospholipids, the other components of the acid insoluble phosphorus, have received little special study and will not be mentioned further here.

Inorganic Phosphorus

Inorganic phosphorus is present in appreciable concentration in the unfertilized egg, and it would be reasonable to suppose that increased needs for phosphate for biosyntheses, energy metabolism, or spicule formation during development might be met by liberation of phosphate from phospholipids or other organic sources. It has now been established, however, that, in the presence of phosphate in the sea water, the developing embryo can augment its content of phosphorus by an increased permeability to the ion, beginning very shortly after fertilization. Although the first observations on the uptake of radioactive phosphate in *Arbacia* eggs made by Brooks (1943) showed only a slight and erratic penetration, and more recently Lindberg (1946) reports the failure of the ion to penetrate, newer studies by Brooks and Chambers (1948), since confirmed by a number of investigators, show that there is indeed a very rapid uptake of the labelled phosphate. Though both unfertilized and fertilized eggs absorb the phosphate and accumulate it over and above the concentration in the ambient sea water, the uptake rate in fertilized eggs is 130 to 160 times as great as for unfertilized.

An example of the time course of accumulation in developing eggs of *Lytechinus pictus* is given in Fig. 1, from Chambers and Whiteley (1949). In this experiment, the uptake of phosphorus by a small sample of eggs in a special thin-walled vessel was followed by continuous recording

with a Geiger counter while sea water containing 0.00043 microcurie of radioactive phosphorus per ml. was flowed over the eggs. The very marked increase in rate of uptake caused by fertilization is well shown, beginning after a lag

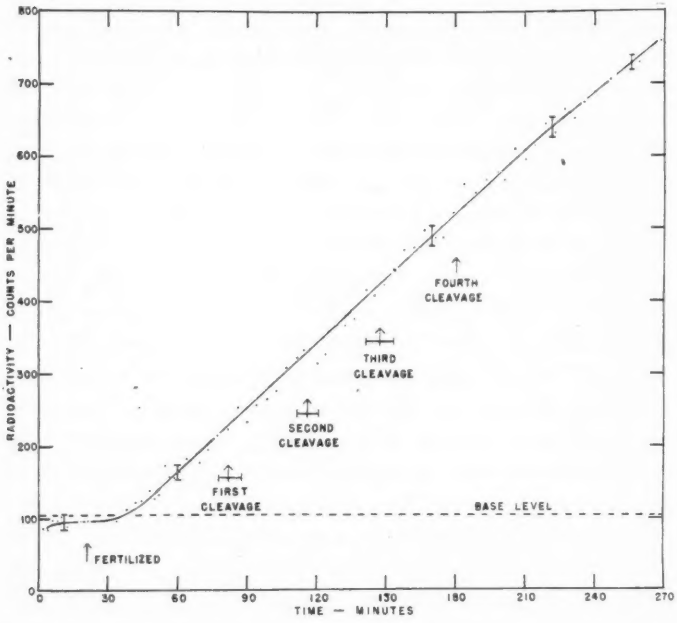

FIG. 1. P^{32} uptake by fertilized eggs of *Lytechinus pictus*. 0.00043 μC/ml. of P^{32} was flowed over the eggs at a rate of about 4 ml./min. at 24.5° to 25.5° C. until cooled at 170 minutes to 21° C. Radioactivity was measured continuously. The base level of activity of the system without eggs is shown by the horizontal line. The vertical bars on the curve indicate twice the standard error. Development of the eggs was 98 per cent. normal to the 128-cell stage.

of 12 to 15 minutes. This lag period corresponds in time with the initial dramatic respiratory changes that are so well known for echinoid eggs, and it is apparent that there is no direct relation between the two events. Similarly,

there is no rhythmic pattern of phosphorus uptake during the mitotic phases, in contrast to the respiratory cycles known for urchin and other kinds of eggs (Zeuthen, 1949). The deviations from a smooth curve are all just within the limit of counting error. Attention was originally called to this lack of inter-relation between uptake and mitosis by Brooks and Chambers (1948).

The distribution of radiophosphorus in unfertilized eggs and in fertilized eggs and cleavage stages is almost entirely among the acid soluble phosphates, the acid insoluble fraction containing only 3.5 to 4.1 per cent. of the total, practically none of which is in the phospholipid material (Chambers, Whiteley, Chambers, and Brooks, 1948). The interesting questions of the distribution of the large acid soluble portion among the components of that fraction and the conditions determining turnover rates there deserve thorough study. Abelson (1948) has made a start in this direction for the eggs of *Arbacia*, finding variable amounts of the isotope in all components of the early embryos.

Though little difference exists between fertilized and unfertilized eggs as concerns the gross chemical distribution of the radiophosphorus, there is a pronounced difference in the cytological distribution. This was determined by bathing the eggs of *Lytechinus pictus* in sea water containing radiophosphorus and then centrifuging them into two parts by the centrifuge technique of Harvey (1941). Radioactivity determinations could then be made on the light fragments separated from the heavy ones. In *L. pictus*, the large, light parts contain an oil cap, a small hyaline layer, the nucleus or mitotic figure, and the yolk, while the smaller, heavier part contains mitochondria and a heavy, optically empty layer. Unfertilized eggs allowed to accumulate radiophosphorus for about 6 hours have the phosphorus distributed so that the lighter part receives 1 to 2.4 times as much as an equal volume of the heavier. Fertilized eggs, however, allowed to accumulate the isotope for 18 to 29

minutes and then centrifuged into two fragments have the isotope distributed between light and heavy fragments in the ratio of 0.48, 0.45 and 0.55 for three experiments. The bulk of the newly absorbed phosphorus in fertilized eggs is associated with mitochondria or the heavy hyaline material.

Propanediol Phosphate

Propanediol phosphate is an ester first isolated and identified by Lindberg (1946) from cow brain. The identification of this ester in echinoderm eggs rests chiefly on the observations that the barium salt of propanediol phosphate is soluble in water and alcohol, which thus sets it apart from most of the heretofore known phosphate esters, and on the fact that it resists both mild acid and alkaline hydrolysis. At least in *S. purpuratus* this substance comprises the largest component of the acid-soluble phosphate. A hypothesis concerning the part played by this substance will be presented later.

Adenosinetriphosphate

The identification of adenosinetriphosphate is rather well established. The determinations of Table 1 are based on the familiar procedure of hydrolyzing the barium insoluble phosphate for 7 minutes at 100° C. in 1 N HCl. Recently, the ultraviolet absorption spectrum of the barium insoluble phosphate of *S. purpuratus* eggs has been measured by the author and is essentially the same as that of adenosinetriphosphate purified from rabbit muscle. These spectra, and that of adenosine for comparison, are shown in Fig. 2, curves *a*, *b* and *c*. When adenosine is deaminated by adenosine deaminase to form inosine, the absorption spectrum is changed as shown in *f* (Kalckar, 1944; Mitchell and McElroy, 1946a). In a like manner the spectra of the sea urchin and rabbit adenosinetriphosphates are changed by the deaminase to those of *d* and *e*. The enzyme was pre-

pared by the procedure of Mitchell and McElroy (1946b). It is also reported by Chambers and White (1948) that they have observed that purified myosin causes hydrolysis

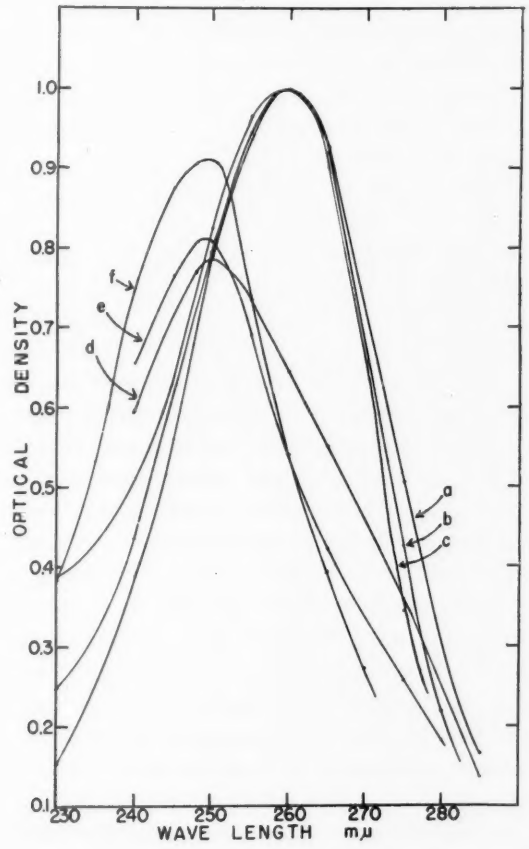

FIG. 2. Absorption spectra of sea urchin (*Lytechinus pictus*) adenosinetriphosphate, *a*, rabbit adenosinetriphosphate, *b*, adenosine, *c*, and the same substances after enzymatic dephosphorylation and deamination, *d*, *e*, and *f*. The adenosine and ATP values have been adjusted to a maximum density of 1.00. The spectra were obtained in 1.15 M phosphate buffer at pH 7.0, with a Beckman spectrophotometer.

of the barium insoluble phosphate from eggs of *Arbacia*, and that actomyosin fibers contract in the presence of this phosphate fraction, just as they do in the presence of adenosinetriphosphate.

It would be of some importance to find a specific tie-in between the energy-rich phosphate of adenosinetriphosphate and developmental processes, either mechanical or chemosynthetic. It is entirely possible, for example, that activation of the egg at fertilization includes an awakening of processes dependent on adenosinetriphosphate, or perhaps mitotic activity is dependent on energy supplied by phosphorylative mechanisms.

Attempts have been made by Runnström (1933) and Lindberg (1943) to find changes in the phosphate balance with fertilization in echinoid eggs which might reflect a change in activity of phosphorylative mechanisms. They report that no appreciable change in any fraction occurred. Barth and Jaeger (1947) have pointed out, however, that analysis for the concentration of a phosphate compound at various times during normal unmodified development might not reveal even large changes in the rate of turnover of the substance because of the likelihood that an increase in its utilization might be balanced by a corresponding increase in its formation, leaving the concentration at any time constant. By interrupting the events leading to adenosinetriphosphate synthesis in *Rana pipiens* gastrulae by imposing, among other things, anaerobic conditions on the embryos, they have been able to show that the adenosinetriphosphate of the eggs falls from 24.6 micrograms/100 embryos to 16 micrograms/100 embryos, concomitant with a partial inhibition of development. The demands of morphogenesis and maintenance are too much for the anaerobic adenosinetriphosphate synthesizing mechanisms. Developmental energy needs during the cleavage stages, however, are less demanding, and the anaerobic mechanisms are able to maintain the adenosinetriphosphate store nearly

intact. These important experiments apparently represent the first demonstration that the energy-rich phosphate bond is closely linked to a developmental process.

Comparable experiments by the author designed to show an interdependence of fertilization and adenosinetriphosphate turnover in the eggs of *S. purpuratus* have so far been inconclusive, and at present the only reported interchange of phosphorus among the acid-soluble components of sea urchin embryos is that found by Zielinski (1939) in *Paracentrotus lividus* embryos poisoned with 0.025 M iodoacetate. In these, a small decrease in inorganic phosphate occurring progressively during the first two cleavages is accompanied by a corresponding increase in a phosphate ester that is probably propanediol phosphate. There was no alteration of adenosinetriphosphate concentration.

It will be remarkable indeed if no more significant interchange of phosphorus occurs in the fertilized echinoderm egg than is indicated by present knowledge in view of the great increase in respiratory activity accompanying activation in the echinoids, the close relation of respiration and mitosis established by Zeuthen, and the increased permeability of the fertilized egg to phosphorus shown by the radioactive isotope studies. Indeed, the mere presence of sizable amounts of adenosinetriphosphate and of an adenosinetriphosphatase to metabolize it (Connors and Scheer, 1947), leads one to expect a role for this ester in these important metabolic changes, though in view of the unusual array of acid-soluble phosphates present, it seems reasonable that the biochemical machinery to produce and utilize the energy-rich bonds may vary considerably from the familiar ones of muscle and yeast.

Attempts to illuminate this biochemical machinery in the echinoid egg have led to the view that the well-known Meyerhof-Embden scheme of muscle plays at most only a minor role. Runnström (1933, 1935) years ago found that neither sodium fluoride nor monoiodoacetate inhibited pro-

duction of the acid of fertilization in sea urchin eggs, and Lindberg (1943) has established that glycogen and glucose breakdown in fertilized eggs or egg homogenates are not blocked by these inhibitors. Since the usual glycolytic mechanisms are inhibited by them, an alternative pathway of oxidation is indicated. This pathway apparently requires phosphorylative mechanisms, however, as Rothschild (1939) has found that phlorizin strongly inhibits the formation of acid of fertilization and, therefore, probably carbohydrate degradation (Lindberg, 1943).

An alternative mechanism to the Meyerhof-Embden scheme is that involving oxidation and decarboxylation of the hexose monophosphate molecule, carbon by carbon, a mechanism suggested by Warburg and Christian (1936) and Lipmann (1936) and developed further by Dickens (1938). Lindberg (1946) has proposed the working hypothesis that this pathway is the predominate one in the sea urchin egg. The suggestion is that hexose monophosphate is oxidized to phosphogluconic acid and this, in turn, to phosphoketogluconic acid. Proponediol phosphate is thought to act in this oxidation. The keto acid is then decarboxylated to form a pentose phosphate. Further oxidation presumably consists of a repetition of these steps.

The hypothesis is based chiefly on the following facts. He has tested the ability of egg homogenates to reduce methylene blue anaerobically in the presence of a number of possible substrates and finds that hexose phosphates, phosphogluconic acid, and propanediol phosphate are the most effective. The corresponding non-phosphorylated substances are ineffective. Further, pentoses increase in amount in the homogenates when propanediol phosphate is added and a small decrease in reducing sugar is found, a fact which could be explained by the oxidative decarboxylation of hexose phosphate. A further involvement of propanediol phosphate in respiratory metabolism is given by Lindberg's observation that the ester can stimulate respiration of the

egg brei by as much as 100 per cent. A complete test of this working hypothesis will be of interest.

In addition to a possible role in energy metabolism in the sea urchin egg, propanediol phosphate and phosphogluconic acid have been found to have a morphogenetic effect in these eggs. Hörstadius and Gustafson (1947) have found both of these esters capable of animalizing isolated animal halves of early embryos of *Psammechinus miliaris* when applied in concentration of about 0.5 mg. per ml. Lactate and pyruvate have identical effects. The effect is expressed as an overdevelopment of the long cilia of the blastula and a suppression of development of the stomodaeum and ciliated band of the fully differentiated halves. Results have not been presented for the effect of the substances on vegetal halves. If Lindberg's hypothesis is correct, these results are in agreement with the suggestion of Lindahl (1936) that animalization is under the control of a gradient of carbohydrate metabolism highest at the animal pole.

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Discussion

Chairman Engle: Thank you, Dr. Whiteley. The occurrence of phosphorus uptake in metabolizing tissues was shown to me for the first time in developing eggs, and there is great interest in this technique. I will now turn this paper over to the conference for discussion.

Dr. Zeuthen: You mentioned that in Barth's work there was a difference between the first period of development and the later divisions in the speed of restoration of ATP. At what time does the difference show up?

Dr. Whiteley: In the amphibian study the early gastrulae were made anaerobic for twenty-four hours, and during this time they started losing ATP and continued to lose it. During the cleavage stages, stage 8, as I remember, the decrease in ATP was considerably less. Apparently the eggs could handle their smaller morphogenetic problems at that time.

Dr. Zeuthen: Nobody has tried to investigate the cleavage period to see if there are differences within the cleavage period?

Dr. Whiteley: I know of no such study. Barth and Jaeger studied an extended period involving several cleavages in the frog egg.

Dr. Haas: As I recall, early experiments of Warburg demonstrate that the respiration of the egg, after fertilization, increases sharply. The respiration can be inhibited by cyanide and it seems to resemble also in every other respect the normal pattern of respiring cells. Wouldn't it

be important then to consider in the developing egg the role of the phosphorylated carbohydrates which are known to participate as intermediates in respiratory and fermentative processes?

DR. WHITELEY: It certainly would be important to look intensively for all the usual phosphorylated intermediates. None of them, other than those discussed, has so far been found in the eggs. One would certainly expect to find more than just these few, but any other phosphate compounds that may be present must be in low concentration, judging from the data of the table presented. Very little acid soluble phosphate is left over after one takes into account the three components discussed.

DR. HAAS: With enzymatic methods it may be possible to detect phosphorylated intermediates which are too unstable or which might be present in too low a concentration to be observed by conventional method. An enzymatic method, for example, is available to determine accurately as little as 10 micrograms of glucose-6-phosphate.

DR. WHITELEY: Enzymatic techniques are certainly the ones to turn to. Glucose-6-phosphate may well be present, but the procedure of analyzing for its barium salt precipitated out of alcoholic solution doesn't give evidence of its presence in this material. Other compounds undoubtedly are also present and their measurement merely waits on the use of sufficiently sensitive techniques. Your enzymatic method for glucose phosphate sounds like a very good one to apply.

DR. SPIKES: Of course it should be pointed out that it is not a general phenomenon that respiration increases after fertilization. In many animals it drops as quickly as it increases in the sea urchin.

CHAIRMAN ENGLE: Are there any other pertinent comments?

DR. CHAMBERS: One of the most striking things about the uptake curve is the extraordinary difference between

the unfertilized and fertilized eggs. It is 160 times as rapid in the fertilized eggs sixty minutes after fertilization than in the unfertilized egg. There was one further question I wanted to ask Dr. Whiteley. We got two milligrams of adenosinetriphosphate per gram of Arbacia egg. I wonder how that would compare with your data. I can't figure it out in my head.

DR. WHITELEY: The agreement between your data and mine is very close, and further, both are in good agreement with the values found for rabbit muscle. Specifically, I find 2.2 mg. of the barium salt of ATP per gram wet weight, or 1.3 mg. of the free acid, in *Strongylocentrotus purpuratus*. The corresponding figures for *Lytechinus pictus* are 3.6 and 2.1. Yields from rabbit muscle run about 3.5 mg./gram wet weight.

CHAIRMAN ENGLE: Going back to this question, I would like to ask Dr. Zeuthen a question. I don't want to presume on your presentation later. Are you going to discuss the role of phosphorus here in relation to oxygen consumption?

DR. ZEUTHEN: No, I am not. I am going to discuss the segmentation period, meaning the period from the first up to the ninth division or so, and the question asked Dr. Whiteley was whether people have bothered with analyzing those periods. Most people are interested in taking samples of what they are interested in over much too long intervals to give any kind of information which can be made, at the present time, to fit into the picture of fertilization.

CHAIRMAN ENGLE: I take it, then, that none of you are committed to a definite statement as to whether the oxygen consumption is definitely correlated with this increase in phosphorus uptake.

DR. WHITELEY: I imagine it would be safe to say there is some correlation somewhere, but how it is related is not known. Not all of the intermediates of the Krebs cycle are known to be present in the echinoderm egg. The metabolic

system here is apparently quite different from that of many other tissues.

DR. TYLER: It certainly would be worth-while to make comparative studies of species, such as Chaetopterus and Cumingia, in which there is a drop in oxygen uptake upon fertilization.

CHAIRMAN ENGLE: Is there any further comment?

DR. WHITELEY: I would like to make a comment in connection with Dr. Moore's talk. He mentioned there were two precursor substances for the fertilization membrane which apparently had to be gotten rid of before the egg can divide. I have tried to block cleavage of echinoderm eggs with anaerobic conditions, but I have been uniformly unsuccessful in getting anaerobic blocks. However, it is true that anaerobic conditions applied at the time of fertilization will block it, and I wonder if the difference in effect of anaerobiosis might not be related to the precursor substances. The failure of the membrane to lift anaerobically would cause retention of the substance by the egg, and therefore prevent cleavage, whereas later the substances would have left the egg, thus possibly making it less sensitive to anaerobiosis.

DR. MOORE: It is a most interesting suggestion from entirely different angles. That seems very reasonable.

DR. SCOTT: I want to ask you what you mean by the statement that the development is not blocked by anaerobic conditions. I agree that it is not entirely blocked by anaerobic conditions, although I know that the common belief is that it is. I have been really working on the cytological aspects of anaerobiosis. I think it is going to take a while to develop a good anaerobic technique. Still we are down to a very low oxygen level. I find mitosis creeping along in the best anaerobic conditions. I would like to know what your criterion was.

DR. WHITELEY: Mainly the delay in cleavage time. I have had uniformly bad luck in causing the first cleavage

to be delayed by anaerobic conditions. Maybe the anaerobic conditions weren't thorough enough, though luminescent bacteria blacked out within a minute under the conditions used. The eggs go ahead and are not delayed.

DR. SCOTT: I must say I get delay. I have passed the nitrogen over hot copper.

DR. WHITELEY: I have used that.

CHAIRMAN ENGLE: Is there any other comment? If not, we will move on to the next paper by Dr. Edward L. Chambers, of the University of California, Berkeley, on "Ionic Exchanges and Fertilization in Echinoderm Eggs."

(Conference concluded in the issue of November–December)